THE BREAKDOWN OF ASPARAGINE, GLUTAMINE, AND OTHER AMIDES BY MICROORGANISMS FROM THE SHEEP'S RUMEN

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[*Manuscript received July* 17, 1963]

Summary

Microorganisms from the rumen of sheep rapidly broke down asparagine, glutamine, nicotinamide, and formamide, with the production of ammonia, but only slowly attacked acetamide and propionamide. Microorganisms from different animals, or collected at different times, had different activities. The results suggested that a separate enzyme or enzymes were involved for each substrate, including perhaps a D- as well as an L-asparaginase. The amide groups of casein were also broken down, but it is uncertain to what extent prior hydrolysis had taken place. While the activities could not be correlated with any morphologically recognizable group of microorganisms, it appeared that asparaginase was mainly associated with the bacteria, glutaminase to a large extent with the protozoa.

The aspartic and glutamic acids formed by deamidation of asparagine and glutamine were further deaminated.

The optimum pH for asparaginase and glutaminase was between 7 and 8, but considerable activity remained even at pH 5.

Extracts from the microorganisms were made. The asparaginase activity in these was inhibited by mercuric ions and to a lesser extent by ammonium and by cyanide ions. No inhibition or activation was found with phthalein dyes, aspartic acid, phosphate, sulphate, or chloride ions, or toluene. The apparent K_m of the asparaginase was less than 10^{-4} M.

I. INTRODUCTION

The natural diet of ruminants contains a considerable variety of non-protein nitrogenous material (Chalmers and Synge 1954), and this includes the amino acid amides asparagine and glutamine. The concentration of these amides varies considerably with the species and conditions of growth of the plant; e.g. ryegrass can contain from *c.* 2 to *c.* 70 mg amide nitrogen per 100 g fresh material (Butler 1951); this corresponds to an intake of, say, 0·1–5·0 g amide nitrogen per sheep per day. Some asparagine and glutamine are also present in combined form in the peptide chain of most proteins. Nicotinamide also is found in most feedstuffs; it is also synthesized by the rumen microbial population (Kon and Porter 1954). Since most of the analytical methods used do not distinguish between nicotinic acid and nicotinamide, it is difficult from published data to be sure of the concentration of the amide. Formamide is produced from histidine by a number of microorganisms (Barker 1961), and this reaction has been shown by Van den Hende, Oyaert, and Bouckaert (1963) to occur in the rumen. Other aliphatic acid amides such as acetamide are not known to occur in natural feedstuffs nor as metabolic products in the rumen.

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Ever since Weiske, Schrodt, and Dangel (1879) found that asparagine supported gain in weight and a positive nitrogen balance in sheep, many workers have looked for satisfactory non-protein nitrogen sources that would replace or spare protein in the diet of ruminants. In many of the early feeding experiments, asparagine was used, but attention gradually turned to other compounds and urea eventually became the main object of research. More recently, some other amides have been tested as nitrogen sources for ruminants. Belasco (1954), using a rather unsatisfactory artificial rumen technique (Warner 1956*a*), found that formamide, acetamide, and propionamide were converted to ammonia and supported growth of, and cellulolysis by, microorganisms. Repp, Hale, and Burroughs (1955) found that formamide and propionamide, given as 15–30% of the nitrogen intake, supported growth in lambs, though formamide was toxic in the concentrations used.

Beauville, Lacoste, and Raynaud (1961) found that asparagine and glutamine served as good nitrogen sources for the growth of mixed bacteria from the rumen and suggested that this was due to the ease of release of ammonia.

None of these workers, however, has studied the breakdown of these amides in the rumen in any detail; this is the aim of the present experiments. In addition, an attempt has been made to identify the sources of the enzymes involved.

Since the breakdown of urea has been extensively investigated by others, it has not been studied here.

II. Materials and Methods

The animals used were mainly English Leicester–Merino ewes or wethers fitted with rumen fistulae. The rations given them are described in Table 1.

For assays of enzyme activity, the substrates were added in solution in water to give a final concentration of 15–20 mM, except for casein (B.D.H. light white casein), which was used at a final concentration of 0·4% w/v (equivalent to 40 mM total nitrogen, 3·3 mM amide nitrogen); with aspartic or glutamic acid, disodium hydrogen phosphate was used to bring the solution to approximate neutrality. When whole rumen liquor was used as enzyme source, it was removed from the animal, strained (Warner 1962*a*), and used without additional buffer at a final concentration of 80% v/v. Fractions of rumen liquor were prepared by appropriate centrifugation, washing once in buffer, and suspending in buffer of volume slightly less than the original rumen liquor. Cell-free extracts were prepared from the deposit obtained by centrifuging strained rumen liquor at 5000 *g* for 30 min at 4°C, with or without prior centrifugation to remove the protozoa. This deposit was slowly poured into 5–10 volumes of acetone at −15°C and allowed to stand at 4°C for 30–60 min. The mixture was then filtered and the deposit washed with acetone, acetone–ether (50%, v/v), and ether, all at −15°C. After drying *in vacuo*, the deposit was extracted with water equal in volume to about one-fifth of the original rumen liquor; the mixture was centrifuged and the supernatant dialysed against distilled water for 24 hr at 4°C. Then an appropriate buffer was added; the final volume was slightly less than that of the original rumen liquor. Incubation of substrate and enzyme was usually for 2 hr at 39°C; experiment showed that liberation of ammonia was linear

TABLE 1

ORIGIN AND CHARACTERISTICS OF RUMEN LIQUOR INOCULA USED IN DEAMIDASE EXPERIMENTS

Inoculum No.	Sheep No.	Date*	Daily Ration†	Total Nitrogen in Rumen Liquor (mg/100 ml)	Microbial Concentrations in Rumen Liquor (numbers/ml):					
					Entodinia $\times 10^{-3}$	Oligotrichs‡ $\times 10^{-3}$	Holotrichs $\times 10^{-3}$	Selenomonads $\times 10^{-6}$	Oscillospira $\times 10^{-3}$	Total Bacteria $\times 10^{-8}$
1	1764	29.viii.62	Pasture	161	220	2	10	480	4600	210
2	1765	5.ix.62	Pasture	196	390	0	20	440	2700	300
3	2753	7.xi.62	Pasture	244	900	20	0	570	1750	350
4	2753	7.ii.63	Pasture	132	400	1	2	260	260	160
5	1593	4.iv.63	Pasture	143	210	4	1	150	900	195
6	1338	2.v.63	1600 g W13	22	240	7	3	70	0	36
7	SO74	18.i.63	700 g R9	82	210	10	20	250	200	150
8	SO74	24.vii.62	700 g F19	163	980	85	45	160	230	160
9	SO74	24.x.62	700 g F19	250	1300	60	45	470	200	370
10	1319	5.ix.62	700 g R9	51	65	1	20	10	10	200
11	MB3	5.ix.62	1000 g E1	80	120	1	1	120	550	250
12	B586	29.viii.62	1000 g M2	228	2600	100	40	0	0	160
13	SO74	7.viii.62	700 g F19	—	810	30	30	320	570	250
14	SO74	7.viii.62	700 g F19	—	510	20	20	260	650	120
15	SO74	7.viii.62	700 g F19	—	510	20	10	270	420	180

* Inocula 1–5 were collected early in the morning; inocula 6–13 were collected just before feeding; and inocula 14 and 15, 2 and 5 hr after feeding respectively.

† The pasture consisted predominantly of *Lolium perenne* and *Trifolium subterraneum*. W13 was a poor quality wheaten chaff. R9 was 50% lucerne chaff, 50% wheaten chaff. F19 was 50% lucerne chaff, 10% linseed meal, 10% coconut meal, 10% oats, 20% wheat. E1 was lucerne chaff. M2 was 50% lucerne chaff, 50% oats. All dry rations were given to the sheep once per day except for the animal providing inoculum 7, where the ration was given in 8 equal batches at 3-hourly intervals.

‡ Excluding the entodinia.

with time under these conditions. For estimation of ammonia and amides, samples were removed and the pH adjusted to about 4 with 1N HCl; after centrifugation at 25,000 *g* for 10 min at 4°C, the supernatant was used for analysis. The values given are corrected for the blank and corrected for any non-enzymic hydrolysis of the substrate.

For pH between 5 and 8, phosphate–acetate buffer (0·1M K_2HPO_4 with the necessary amount of glacial acetic acid added) was used. For pH between 7 and 9, 0·05M Tris–HCl buffer (Gomori 1946) was used. When testing the action of phosphate and chloride ions on cell-free extracts, 0·05M sodium borate (pH after addition of extract, etc. slightly less than 9) was used. All pH values were measured at 39°C.

Ammonia was estimated by a modification of the vacuum distillation technique of Pucher, Vickery, and Leavenworth (1935). A sample containing 1–20 μmoles ammonia nitrogen in 10 ml water was put in a 500-ml round-bottomed flask and 5 ml of the phosphate–borate buffer added; the pH was then adjusted to 9·5±0·3 with the borate–sodium hydroxide solution. The flask was then connected, through a condenser and a receiving flask containing 3 ml of the boric acid indicator solution of Conway and O'Malley (1942), to a vacuum pump and manometer. The pressure was lowered to 35–40 mm Hg, air that had been scrubbed in dilute sulphuric acid was admitted through an air leak to the test solution at a rate of about 2–3 bubbles per second, and the flask immersed in a water-bath at 40–42°C. After distillation for 15 min the receiving flask was removed and its contents titrated with 0·01N hydrochloric acid. This technique was shown to give 97–100% recovery of ammonia nitrogen without breaking down the labile amides such as glutamine or formamide.

Amides were estimated by hydrolysing after the technique of Lewis *et al.* (1950) and estimating the liberated ammonia as above.

Total nitrogen was estimated by a microKjeldahl procedure: the solution was digested according to the technique of McKenzie and Wallace (1954) and distilled in the apparatus of Steyermark *et al.* (1951) into 2 ml of the boric acid indicator solution of Conway and O'Malley (1942), which was then titrated with 0·01N hydrochloric acid.

For paper chromatography, filter paper disks in an apparatus similar to that of Proom and Woiwod (1951) were used at a temperature of 39°C. For the separation of aspartic acid and asparagine, or of glutamic acid and glutamine, ethanol–ammonium hydroxide–water (19 : 1 : 5 v/v) was used; incubation for 25 min was required. For the separation of all four substances, a freshly prepared mixture of phenol–ammonium hydroxide–water (30 : 0·1 : 10 w/v) was found satisfactory with an incubation time of 45 min. After development, the papers were dried at room temperature and sprayed with a solution of 0·2 g isatin in 70 ml ethanol, 8 ml acetic acid, and 1·6 ml ammonium hydroxide. They were then placed in an oven at 105°C for 4 min. This reagent was either more or very little less sensitive than ninhydrin for all amino acids tested, and gave a wide variety of colours with the individual amino acids.

Microbial counts were made by the technique of Warner (1962*a*). Sufficient cells were counted to give 95% confidence limits of about ±20% where the quoted value is greater than about 50; accuracy was less for the lower counts.

TABLE 2

DEAMIDATION ACTIVITY OF DIFFERENT SAMPLES OF RUMEN LIQUOR AGAINST DIFFERENT AMIDE SUBSTRATES

Inoculum No*	L-Asparagine		DL-Asparagine Activity†	L-Glutamine		DL-Glutamine Activity†	Nicotinamide Activity†	Formamide Activity†	Acetamide Activity†	Propionamide Activity†
	Deamidase Activity†	Deamination‡		Deamidase Activity†	Deamination‡					
1	6·5	100	—	2·9	80	—	—	—	—	—
2	13·2	70	—	6·2	50	—	—	—	—	—
3	13·4	40	—	5·8	50	—	—	—	—	—
4	4·5	90	5·5	2·0	70	1·9	5·4	10·8	0	0·9
5	9·9	60	7·0	3·7	40	3·3	5·7	9·8	0·2	1·5
6	1·1	—	1·1	0·2	—	0·2	0·7	0	0	0
7	2·9	70	3·2	1·6	30	1·2	4·1	1·5	0	0·4
8	7·7	40	—	3·3	0	—	—	—	—	—
9	8·5	50	—	2·6	100	—	—	—	—	—
10	8·3	30	—	2·0	0	—	—	—	—	—
11	4·7	50	—	1·3	50	—	—	—	—	—
12	3·0	70	—	0·9	100	—	—	—	—	—

* See Table 1 for description.

† μMoles amide nitrogen disappearing per 100 ml per minute.

‡ The percentage by which the ammonia nitrogen appearing exceeded the amide nitrogen disappearing (see text).

III. Results

(a) *Deamidase Activity of Different Inocula*

All those amides expected to be found naturally in the rumen were broken down when incubated with rumen liquor from a number of animals on a variety of diets (Tables 1 and 2). Ammonia nitrogen appeared in amounts at least equal to the amide nitrogen disappearing, so that the major route of breakdown was by deamidation. This deamidase activity varied in the one animal from period to period, as shown by the results with inocula 3 and 4 (Table 2), inocula 8, 9, and 13 (Tables 2 and 3), and other unpublished experiments. The activity did not appear to depend on the presumed nitrogen or amide content of the feed, but did show some tendency to vary with the total nitrogen content of the rumen liquor inoculum. Deamidation of acetamide and propionamide, not expected to be normal substrates, was very slow. Deamidation of

Table 3

Effect of feeding on deamidase activity of rumen liquor

Inoculum No.*	Time after Feeding (hr)	Substrate: L-Asparagine		Substrate: L-Glutamine	
		Deamidase Activity†	Deamination‡	Deamidase Activity†	Deamination‡
13	0	4·6	100	1·9	40
14	2	3·8	30	0·3	0
15	5	1·6	90	0·2	0

* See Table 1 for description.

† μMoles amide nitrogen disappearing per 100 ml per minute.

‡ The percentage by which the ammonia nitrogen appearing exceeded the amide nitrogen disappearing.

DL-glutamine was usually a little slower than that of L-glutamine, suggesting that perhaps the D-glutamine was inhibiting the activity; the experiments were terminated before half the amide had been hydrolysed, so it is not known if D-glutamine was attacked. On the other hand, in the case of inoculum 5 at least, some of the D-asparagine must have been hydrolysed; deamidation of DL-asparagine was sometimes more rapid than that of L-asparagine, so that no inhibition was occurring.

Both asparaginase and glutaminase activities decreased shortly after feeding (Table 3), the glutaminase activity being particularly reduced.

Since the ratios of the activities against the different substrates varied from inoculum to inoculum, it is probable that different enzymes and perhaps different organisms were involved. Possible explanations of the differing ratios of activity against L-asparagine to that against DL-asparagine could be the existence of two enzymes, L-asparaginase and D-asparaginase, or perhaps L-asparaginase and a racemase.

An extract of the pasture plants being eaten by the grazing animals was tested and shown to contain no deamidase activity against L-asparagine or L-glutamine

under these conditions, so that the enzymes must come from the animal or its microorganisms.

(*b*) *Deamidation of Protein*

When rumen liquor was incubated with casein, a large amount of ammonia appeared (Table 4). It is assumed that the protein was hydrolysed and the liberated amino acids deaminated (Warner 1956*b*). Since the amide nitrogen was only about 8% of the total nitrogen of the casein, a large production of ammonia from amino groups would render impossible the detection of a small breakdown of amino groups and make the determination of even a considerable breakdown inaccurate; in experiments with rumen liquor from a grazing sheep, some diminution in the amide

TABLE 4

AMMONIA PRODUCTION FROM CASEIN AND FROM L-ASPARTIC ACID AND L-GLUTAMIC ACID AND THEIR AMIDES BY RUMEN LIQUOR AND WASHED SUSPENSIONS OF RUMEN BACTERIA

Substrate	Whole Rumen Liquor*		Washed Bacterial Suspension†	
	Decrease in Amide Nitrogen (μmoles/100 ml/min)	Increase in Ammonia Nitrogen (μmoles/100 ml/min)	Decrease in Amide Nitrogen (μmoles/100 ml/min)	Increase in Ammonia Nitrogen (μmoles/100 ml/min)
Casein	0·9	11·2	1·9	3·0
L-Asparagine	13·0	18·5	11·6	15·3
L-Aspartic acid	—	9·4	—	3·4
L-Glutamine	6·7	8·6	3·1	3·4
L-Glutamic acid	—	1·5	—	0·4

* Collected from sheep No. 2753 on pasture.

† Prepared from rumen liquor collected from sheep No. SO74 (ration: 700 g R9/day; see Table 1). The protozoa and larger feed particles were removed by centrifugation at 200 *g* for 3 min, then the bacteria were collected by centrifugation at 25,000 *g* for 10 min, washed once in phosphate–acetate buffer, pH 7, and suspended in buffer of volume slightly less than that of the original rumen liquor.

nitrogen of casein was found (Table 4), but when rumen liquor from the pen-fed sheep SO74 was used, no deamidation could be detected. However, washed suspensions of rumen bacteria from sheep SO74 showed considerably diminished deaminase activity and allowed the breakdown of the amide groups of casein to be clearly demonstrated (Table 4). In this experiment, 90% of the casein nitrogen was rendered soluble in 5% trichloroacetic acid, so that it remains uncertain whether the amide groups were attacked in the native protein or only after some measure of hydrolysis.

(*c*) *Microorganisms Responsible for the Deamidase Activity*

Comparison of Table 1 with Tables 2 and 3 showed no correlation between the deamidase activity and the concentration of any morphologically recognizable group of microorganisms. It remains possible, of course, that one or a few species, not morphologically distinguishable from inactive types, provided all or most of the activity.

That deamidase activity is not a universal property of the microorganisms of the rumen is shown by tests of fractions of rumen liquor separated by differential centrifugation. By this means, bacteria free from protozoa can be obtained, but protozoa free from bacteria cannot, owing to adherence of some bacteria to the surface of the protozoa. In these experiments, it was thought undesirable to subject the organisms to prolonged washing; consequently, about one-quarter of the bacterial numbers remained with the protozoa, and, in the third experiment shown in Table 5, only rough separation of the bacteria into large, medium, and small organisms was obtained; the supernatants were substantially free of microorganisms. Nevertheless, considerable fractionation of the deamidase activity was found (Table 5). Very little activity was found in the supernatant liquid, in conformity with the general finding that little enzyme activity of any sort is found free in solution in rumen liquor; this also implies that the enzymes are produced by the microorganisms, not the host animal. Most of the asparaginase activity was associated with the bacteria, particularly with medium-sized bacteria. On the other hand, a large part of the glutaminase activity was associated with the protozoal fraction, though the possibility that this was due to contaminating bacteria cannot be completely ruled out. Very little activity was associated with the very small bacteria.

(*d*) *Deamination*

In most experiments in which asparagine and glutamine were used, the increase in ammonia nitrogen in the reaction mixture was greater than the corresponding decrease in amide nitrogen. This was interpreted as due to further deamination of the amino acid produced by the deamidation.

That this interpretation was correct is shown firstly by the ability of the inocula to deaminate the free amino acids (typical results are shown in Table 4), and secondly from paper chromatography experiments. Rumen liquor incubated with asparagine showed the presence of aspartic acid, in considerable concentration if the extent of the apparent deamination was low, but in traces only if it was high; comparable results were found with glutamine.

The approximate extent of this deamination, calculated as the percentage of excess ammonia nitrogen appearing relative to the amide nitrogen disappearing, is shown in Tables 2, 3, and 5. There was a tendency for a high rate of deamidation to be accompanied by a low deamination and *vice versa*; this would suggest that, in these specimens of rumen liquor, there was less variation in the rate of deamination than in the rate of deamidation.

(*e*) *pH Optima*

Washed suspensions of rumen bacteria and cell-free extracts from the bacteria or the whole microbial population, from several sheep, were tested over the pH range 5–9 against both asparagine and glutamine. A broad pH optimum was found, lying between 7 and 8 in both cases. There was comparatively little diminution of activity on either side of the optimum; the activity at pH 5 was more than 30%, at pH 6·5 70–85% in different experiments, and at pH 9 about 90% of the maximum activity.

TABLE 5

DEAMIDASE ACTIVITY OF DIFFERENT FRACTIONS OF RUMEN LIQUOR

Sheep No.	Daily Ration*	Fraction of Rumen Liquor	Sedimentation Conditions of Suspension†	Total Nitrogen (mg/100 ml)	Substrate: L-Asparagine		Substrate: L-Glutamine	
					Deamidase Activity‡	Deamination§	Deamidase Activity‡	Deamination§
1319	700 g R9	Whole	—	51	6·1	40	—	—
		Protozoa	100 *g*, 3 min	20	0·4	100	—	—
		Bacteria	25,000 *g*, 10 min	19	2·5	40	—	—
		Supernatant	—	12	0	—	—	—
2753	Pasture	Whole	—	285	11·8	80	2·9	100
		Protozoa	250 *g*, 3 min	152	3·6	50	2·1	80
		Bacteria	25,000 *g*, 10 min	120	8·2	80	0·9	100
		Supernatant	—	36	0·2	—	0	—
2753	Pasture	Whole	—	227	10·6	80	4·1	50
		Protozoa	100 *g*, 3 min	73	2·2	20	2·2	20
		Large bacteria	500 *g*, 5 min	37	1·8	10	0·7	0
		Medium bacteria	2,500 *g*, 20 min	38	7·0	40	1·8	0
		Small bacteria	40,000 *g*, 15 min	24	0·5	—	0·1	—
		Supernatant	—	33	0·5	—	0·1	—

* Rations are described in Table 1.

† Whole rumen liquor was differentially centrifuged as indicated until the final supernatant was obtained. Deposits were washed once and resuspended in phosphate–acetate buffer, pH 7, slightly less in volume than the original rumen liquor volume.

‡ μMoles of amide nitrogen disappearing per 100 ml per minute.

§ The percentage by which the ammonia nitrogen appearing exceeded the amide nitrogen disappearing.

(*f*) *Deamidase Activity and Its Inhibition in Cell-free Extracts*

Extracts of microbial cells from samples of rumen liquor with high deamidase activity showed no measurable activity against formamide, acetamide, propionamide, or nicotinamide; low activity (0·2–0·5 μmole ammonia released per 100 ml per minute) against L-glutamine and casein; and moderate activity (1·5–4·0 μmole ammonia released per 100 ml per minute) against L-asparagine. This is further evidence that different enzymes are involved in the breakdown of different amides. The rate of breakdown of both L-asparagine and L-glutamine varied linearly with the concentration of the extract. The deaminase activity of these preparations was negligible.

Deamidation of L-asparagine was almost completely inhibited by 1 mM mercuric chloride when 15 mM L-asparagine in 0·05M borate buffer, pH 9, was used as substrate for a preparation able to release about 1·5 μmoles ammonia per minute per 100 ml from asparagine; 5 mM ammonium sulphate inhibited 50% of the activity; and 100 mM potassium cyanide inhibited about 10% of the activity. No inhibition or activation was found when bromocresol green, bromocresol purple, Evans blue, or phenolphthalein (all 1 mM), aspartic acid, dipotassium hydrogen phosphate, sodium sulphate, or sodium chloride (all 100 mM), or saturated toluene were used.

The small glutaminase activity of the same preparation was not inhibited by bromocresol green; no other investigations were made of this activity.

In order to examine further the action of ammonium sulphate on the asparaginase activity, an attempt was made to investigate the kinetics of this asparaginase, though it was realized that it was probable that the activity measured was due to enzymes from several organisms, and hence possibly different in properties. It was found that the "enzyme" had a high affinity for L-asparagine, there being little diminution in the rate of hydrolysis at 0·2 mM concentration, so that the Michaelis constant was probably considerably less than 10^{-4} M. The techniques used here for the estimation of ammonia were inadequate for such low concentrations and no further investigation was made.

IV. Discussion

The very slow breakdown of propionamide and, particularly, acetamide in these experiments contrasts with their apparently rapid breakdown inferred from the experiments of Belasco (1954) and Repp, Hale, and Burroughs (1955). In both these cases, however, there was ample time for the enrichment of those organisms which attack the amides and for the induction of the necessary enzymes if they are not constitutive. Repp, Hale, and Burroughs (1955), indeed, found that the breakdown *in vitro* of propionamide by microorganisms from the rumen of an animal fed a ration not containing propionamide was slow, and that the longer the host animal had been fed propionamide, the more rapid the release of ammonia from that amide by the rumen microorganisms *in vitro*.

In order to show to what extent hydrolysis of a protein is necessary before deamidation can take place, it would be necessary to discover a method of inactivating the proteases without harm to the deamidases, or, alternatively, to find a protein

not susceptible to the proteases, since the mixed microbial population in the rumen always shows some proteolytic activity (Warner 1956*b*).

The considerable diminution in deamidase activity, particularly glutaminase activity, after feeding is somewhat puzzling. Part would be due to the drop in pH and part to the decline in concentration of most microorganisms in strained rumen liquor after feeding (Warner 1962*b*), due presumably to dilution by saliva and perhaps by influx of water across the rumen wall. These factors alone, however, could not account for all the drop in glutaminase activity. Perhaps the glutaminase activity was associated more with those rumen microorganisms "fixed" to feed particles rather than to those "free" in solution, though this is not consistent with the finding that glutaminase activity is strongly associated with the protozoal fraction of rumen liquor.

TABLE 6

RATES OF BREAKDOWN OF NITROGENOUS COMPOUNDS IN THE RUMEN OF THE SHEEP

Substance	Assumed Intake of Nitrogen into Rumen* (g/day)	Reaction	Maximum Rates of Breakdown (g nitrogen/day)	Reference
Protein	20 in feed	Hydrolysis	36	Warner (1956*a*)
Amino acids	1 in feed; 20 from protein	Deamination	18	Warner (1956*b*)
Combined amides	1 in protein	Deamidation	2	This work
Free amides	1 in feed	Deamidation	20	This work
Nitrates	0·5 in feed	Reduction to ammonia	3	Lewis (1951)
Nucleic acids and derivatives	1 in feed and microorganisms	Deamination	1·7‡	Jurtshuk, Doetsch, and Shaw (1958)
Urea	3 from saliva and blood	Hydrolysis	135	Carroll (1962)

* Nearly all values are subject to considerable variation in special circumstances.

† As described in the literature, extrapolated to a rumen volume of 5000 ml and a duration of 24 hr.

‡ Rate for xanthine.

The active deamination of glutamic and, particularly, aspartic acids formed from glutamine and asparagine is consistent with the findings of Sirotnak *et al.* (1953), Lewis (1955), and Looper, Stallcup, and Reed (1959) that aspartic acid is rapidly and glutamic acid more slowly broken down by rumen microorganisms.

The optimum pH for the asparaginase and glutaminase activities found in these experiments is similar to that found for these enzymes from several other sources, though other different enzymes are known (Zittle 1951; Roberts 1960; Varner 1960). The deamidase activities of *Mycobacterium phlei* are somewhat similar to those of the rumen organisms, there being rapid breakdown of formamide, nicotinamide, and asparagine, and slow breakdown of glutamine, but there was also moderate hydrolysis of acetamide and none of D-asparagine (Halpern and Grossowicz 1957). The *M. phlei* L-asparaginase showed a sharp drop in activity below

pH 7, its K_m was about 5×10^{-3}M, and it was inhibited by the D-isomer. *Brucella abortus* has stereo-specific enzymes for L- and D-asparagine (Altenbern 1955). Yeast asparaginase is inhibited by mercuric but not by cyanide ions, much like the rumen enzymes (Grassmann and Mayr 1933). Glutaminase from dog kidney is activated by phosphate and sulphate, but inhibited by chloride and cyanide ions, also by phthalein dyes (Roberts 1960). The presumed K_m for asparaginase found in the present experiments is lower than others reported elsewhere.

The relative ease of extraction into solution and the lack of sensitivity to toluene make the rumen asparaginases and glutaminases studied here more similar to the rumen proteases than the rumen amino acid deaminases (Warner 1956*b*; Desbals and Raynaud 1961).

Any amide in the ration escaping this rapid breakdown by the rumen micro-organisms might still be hydrolysed by the gastric mucosa; Zalesskaya, Martinson, and Tyakhepyl'd (1961) found that both dog and cat gastric mucosa hydrolysed asparagine and, sometimes, glutamine.

It is now possible to give values for the probable rates of breakdown of most nitrogenous compounds in the rumen, as shown in Table 6. These values can be compared with an estimated maximum rate of ammonia uptake by the rumen microorganisms of 24 g nitrogen per day (Bloomfield, Garner, and Muhrer 1960).

Except for the hydrolysis of urea and perhaps the deamidation of free amides, the rate of breakdown of these compounds is similar to the rate of intake. Since all these rates are subject to considerable variation, it can be seen that relatively minor changes of either could lead to the rate of intake exceeding the rate of breakdown, so that substantial amounts of the substance concerned could pass down the intestinal tract or be absorbed into the blood, though this is unlikely to happen with urea unless very large doses are given. Similarly, the rate of production of ammonia, particularly from urea, could exceed the rate of uptake by the microorganisms, leading to the accumulation of ammonia in the rumen and its absorption into the blood. All these effects are, in fact, known to occur.

V. Acknowledgments

I wish to thank Dr. J. P. Hogan and Mr. R. H. Weston for access to their sheep and Mr. K. Ayers for technical assistance.

VI. References

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